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Isolation and Characterization of Human Monoclonal Antibodies Against Hepatitis C Virus Envelope Glycoproteins

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The isolation and characterization of human monoclonal antibodies (humAbs) against the hepatitis C Virus (HCV) glycoproteins E1 and E2 are described. B-cells from blood donors with anti-HCV were transformed with Epstein-Barr virus. The supernatants of the resulting lymphoblastoid clones were screened by ELISA with an extract of cells infected with a recombinant vaccinia virus RMPA95 expressing the envelope proteins E1 and E2 of an HCV genotype 1a virus (H strain). Positive clones were fused to the heteromyeloma cell line K6H6/B5. Fifteen heterohybridoma cell lines have been established. The specificity of the isolated humAbs was determined both by ELISA and Western blot assays. Several recombinant extracts expressing either the E1 or E2 protein or truncated forms were used in an attempt to map the epitopes on the viral glycoproteins. Some of the humAbs were used successfully for immunofluorescence investigation of transfected cells. Seven specific anti-E2 humAbs, which react with the envelope protein 2 of genotype 1a and 1b isolates, were characterized. ***J. Med. Virol. 55:28–34, 1998.***

KEY WORDS: hepatitis; monoclonal antibodies; recombinant antigens; vaccinia virus; baculovirus; immunofluorescence

INTRODUCTION

Hepatitis C virus (HCV) is an enveloped virus whose genetic information is encoded in a 9.4-Kb positive strand RNA molecule. A highly conserved noncoding region of 341 bp is localized at the 5′-end of this viral genome, which is followed by a long open-reading frame coding for a polyprotein of approximately 3,010 amino acids [Matsura and Miyamura, 1993]. Two putative envelope glycoproteins, E1 (gp35) and E2 (gp72), have been identified, with 5 or 6 and 11 N-linked glycosylation sites, respectively [Dubuisson et al., 1994]. A high level of genetic variability is associated with the envelope genes. This is highly accentuated at the 5′-end of the E2 gene, where two hypervariable regions, termed HVR1 and HVR2, have been described [Kato et al., 1992]. Serological studies, using recombinant proteins and synthetic peptides, suggest that the genetic variability of the HVR1 may be due to immune selection exerted by neutralizing antibodies [Kato et al., 1993; Weiner et al., 1992]. This interpretation has been strongly supported by the absence of such a variability in an agammaglobulinemic patient who was followed up for two and a half years [Kumar et al., 1994].

HCV envelope antigens are highly immunogenic when expressed in glycosylated forms [Lanford et al., 1993]. Preliminary data suggest the existence of conserved epitopes within the E2 protein [da Silva Cardoso et al., 1997; Lesniewski et al., 1995]. Recent work proposes the existence of neutralizing antibodies in serum from infected patients [Rosa et al., 1996; Zibert et al., 1995; Zibert et al., 1997]. Therefore, for therapeutic purposes, it would be of great significance to identify neutralizing determinants that remain conserved among different HCV genotypes. The objective of this study was to assess the immune response of infected individuals to the HCV-E1/E2 antigens. For this purpose, cell lines producing monoclonal antibodies capable of binding to glycosylated forms of recombinant HCV envelope proteins were established. These clones were derived from human B-cells isolated from anti-HCV positive blood donors. Relevant epitopes were identified that may be useful for the development of

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therapeutic strategies. The monoclonal antibodies themselves might be used as specific reagents for the isolation and characterization of recombinant proteins, for immunohistology, and, ultimately, for passive immunization.

MATERIALS AND METHODS

Peripheral Blood Mononuclear Cells (PBMC) Donors

PBMC donors were selected through routine screening of blood donors. Donors who were reactive in a third-generation ELISA (Ortho Diagnostic Systems, Grenzach-Wyhlen, Germany) and who also tested positive with a supplemental test (RIBA or Matrix, Ortho or Abbott, respectively) were analyzed for anti-E1/E2 by ELISA. From previous investigation it is known that 76% of the anti-HCV positive donors in the investigated population are infected with genotype 1b [Cardoso et al., 1996]. Sera from anti-HCV positive and negative blood donors were used as controls.

Human Monoclonal Antibodies

Cell culture. Human monoclonal antibodies (humAbs) were generated by Epstein-Barr virus transformation of PBMC from HCV-infected blood donors [Siemoneit et al., 1994]. Anti-HCV antibody producing lymphoblastoid cells (LCL) were fused with the heteromyeloma cell line K6H6/B5 as described [Carroll et al., 1986]. Positive heterohybridomas were cloned three times by limiting dilution and finally expanded for antibody production.

Antienvelope screening. Cell culture supernatants were screened for specific anti-E1/E2 antibodies by ELISA. Extracts of cells (BHK 21) infected with either the recombinant E1/E2 vaccinia virus RMPA95, or wild-type virus (no insert), were kindly provided by Dr. Teresa Cabezon (SmithKline Beecham Biologicals, Belgium). The cloned DNA fragment originated from an isolate of 1a genotype (H strain) and covered the complete E1, E2, and p7 genes as well as a portion of NS2. Expression of this insert resulted in processed and glycosylated E1 and E2 proteins. Microtiter plates were incubated overnight at 4°C with 0.25 μg/well Lectin from Galanthus nivalis (GNA). Plates were washed three times with phosphate buffered saline (PBS) containing 0.1% Tween 20 (washing buffer) and blocked for 60 minutes at 37°C with 1% bovine serum albumin (BSA) in PBS. Subsequently, plates were washed three times with washing buffer. The recombinant or wild-type extracts were added to the plates at 7 μg/well in PBS with 1% BSA, 1% fetal calf serum (FCS), and 0.1% Tween 20 (incubation buffer) and incubated either for two hours at 37°C or overnight at 4°C.

Removal of unbound proteins was attained by washing the plates three times with washing buffer. Cell culture supernatants were diluted 1:2 in incubation buffer and incubated for 60 min at 37°C. Plates, again washed three times, were then incubated with biotinylated goat anti-human IgG (Dianova, Hamburg, Germany) at a 1:5,000 dilution in incubation buffer for 60 min at 37°C. Plates were washed three times and incubated with streptavidin–peroxidase conjugate at a 1:2,000 dilution for 30 min at 37°C. Five washing steps followed. Binding was visualized using the 0-phenylenediamine dihydrochloride (OPD) reaction and measured at 492 nm in a microtiterplate reader. The cutoff value was calculated by multiplying the mean adsorbance of negative sera, which were tested simultaneously by a factor of three. The humAbs UL/F35 (anti-HLA class I) and UL/F33 (anti-HCVE1) were used as controls [Siemoneit et al., 1995; Wölpl, personal communication].

Antibody purification and labeling. Heterohybridoma clones were grown in IgG-depleted FCS (Gibco, Eggenstein, Germany) and IgG humAbs were isolated from the supernatant by a one-step procedure using a HiTrap protein G column, according to the manufacturer's instructions (Pharmacia Biotech, Freiburg, Germany). Purified IgG antibodies were dialyzed against PBS and stored frozen at a concentration of 0.5 to 1.0 mg/ml. Aliquots of the preparations were labeled with digoxigenin, following the instructions of the DIG-Antibody Labeling Kit (Boehringer Mannheim, Mannheim, Germany).

Competitive ELISA. ELISA plates were treated with GNA, blocked with BSA, and coated with GNA-captured glycoproteins contained in the recombinant extracts, as described above. Test sera were added to the wells, at a 1:10 dilution in incubation buffer, and incubated at 37°C for 90 min. Plates were then washed three times with washing buffer. Digoxigenin-labeled humAbs were then added at a 1:300 dilution in incubation buffer and plates were incubated at 37°C for 60 min. This was followed by three washing steps. Sheep antidigoxigenin–peroxidase-conjugated Fab fragments (Boehringer Mannheim) at a 1:1,000 dilution in incubation buffer were added and incubated for 60 min at 37°C. Five washing steps followed. Visualization was carried out as described above. Competition capacity was calculated as follows: [OD492mAb with competitor]—[OD492mAb without competitor]. The cutoff value was defined as three times the mean adsorbance of the negative sera tested in the same plate.

Denaturation Experiments

Vaccinia recombinant E1/E2 extract was denatured either by boiling for 5 min at 95°C in 0.1% sodium dodecyl sulfate (SDS) or boiling for 5 min at 95°C in 0.1% SDS and 50-mM 1,4-dithiothreitol (DTT). The denatured extracts were used as solid-phase antigens in ELISA assays as described above.

Characterization of Epitopes

Anti-E1/E2 humAbs were characterized using extracts from Sf9 insect cell cultures infected with recombinant baculoviruses. The cloned DNA fragments originated from a genotype 1b isolate, and were kindly provided by Dr. T. Miyamura (NIH, Japan). The three recombinant vectors either encoded E1 (Ac816), E2 (Ac1327), or E1/E2 (Ac827) as a fusion protein. Reactivity was measured by ELISA as described above for the vaccinia extracts. Alternatively, humAbs were

characterized using extracts from BHK 21 cells infected with recombinant vaccinia viruses RTRM7 and RTRM4, carrying the E1 or E2 DNA fragment, respectively. These DNA fragments originated from a genotype 1b isolate. The recombinant vaccinia extracts were kindly provided by Dr. Teresa Cabezon (Smith-Kline Beecham Biologicals).

Supernatants of transfected CHO cells secreting a recombinant fusion protein of the Herpes simplex virus envelope protein gD and HCV-E2 (gD/E2), or a truncated form of HCV-E2 (gD/E2t) harboring a deletion of 147bp at the 3′-end of the E2-insert, were also used to characterize the isolated antibodies. These supernatants as well as a negative control supernatant (expressing the HSV gD protein) were kindly provided by Ms. Ki Jeong Lee (Pohang University, Korea).

Immunoblotting

Western blot analysis was performed using the recombinant extracts described above. Electrophoresis and transfer to nitrocellulose membranes were performed according to standard protocols under nonreducing and nondenaturating conditions [Laemmli, 1970; Towbin et al., 1979]. Extracts were loaded in buffer containing 125-mM Tris, 20% Glycerol, and 0.1% SDS. Running buffer contained 0.2-M Glycine, 25-mM Tris, and 0.1% SDS. Membranes were blocked overnight with 1% blocking reagent from Schleicher & Schuell in PBS. Purified primary antibodies were tested at a dilution of 1:1,000 in blocking reagent, whereas supernatant of hybridoma cultures were tested at a dilution 1:2 in the same reagent. Bound primary antibody was detected using a monoclonal antihuman IgG (Fc-specific) biotin conjugate (Sigma Immuno Chemicals, Deisenhofen, Germany). Color reaction occurred after streptavidin and biotinylated alkaline phosphatase incubation steps with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, BCIP/NBT (Bio-Rad, München, Germany).

Immunofluorescence Microscopy

A continuous human cell line expressing by induction the structural region of a genotype 1b HCV isolate [Moradpour et al., manuscript in preparation] was used for indirect immunofluorescence microscopy essentially as described [Moradpour et al., 1996]. In brief, cells were fixed with 2% paraformaldehyde, permeabilized with 0.05% saponin, and incubated for 1 hr with humAbs at a concentration of 2 μg in 100-μl PBS containing 3% BSA and 0.05% saponin. Bound humAbs were detected after incubation with fluorescein isothiocyanate-conjugated rabbit F(ab')2 fragment against human immunoglobulins (Cappel, Durham, NC). Cells not expressing HCV protein served as control for specificity of the staining reaction.

RESULTS

Over 50 LCLs producing antibodies that bind to recombinant RMPA95 were identified by ELISA from 14 different blood donors. None of these antibodies reacted with extracts from cells infected with wild-type vaccinia virus. Selected LCLs were fused with the heteromyeloma cell line K6H6/B5 in order to obtain stable monoclonal cell lines. This approach yielded 15 antibody-producing heterohybridomas. All 15 purified monoclonal antibodies were further characterized as being of IgG1 subtype and containing a kappa light chain. To ascertain whether these humAbs bound to linear or conformation-dependent epitopes, denaturation experiments were carried out. The recombinant E1/E2 antigen was either denaturated by boiling in an SDS solution or by boiling in SDS and DTT (see Materials and Methods above). Antibodies were tested in parallel by ELISA with native and denatured antigen. In this case, the untreated native E1/E2 extract was used as a positive control. Figure 1 shows the two main patterns of reactivity obtained with humAbs. The antibody 2H1, for instance, did not bind to the boiled antigen. However, humAb 4F7 binding was only interrupted after denaturation of antigen in the presence of DTT, a reducing reagent that breaks down disulfide bonds. Approximately 40% of the isolated humAbs behaved like 2H1, while the other 60% showed a reaction pattern identical to that of 4F7. In an attempt to localize the epitopes recognized by the different humAbs, they were assayed by ELISA with extracts originating from both recombinant baculoviruses and vaccinia viruses containing the individual envelope genes from an HCV isolate of genotype 1b. As shown in Figure 2, the humAbs 4F7, 1H3, 2G1, 4E5, 4D2, and 4F1 reacted with the E2 antigen expressed in insect cells. Specificity of the assay was demonstrated by the negative reaction of the anti-HLA UL/F35, as well as by the positive reaction of the anti-E1 monoclonal UL/F33 with both vaccinia and baculovirus recombinant E1 extracts. Only a moderate reaction could be observed between humAbs and the recombinant vaccinia extract RTRM4 that contains the E2 glycoprotein. The humAb 4F7, which showed a strong reactivity to both genotypes in ELISA assays, was further tested in Western

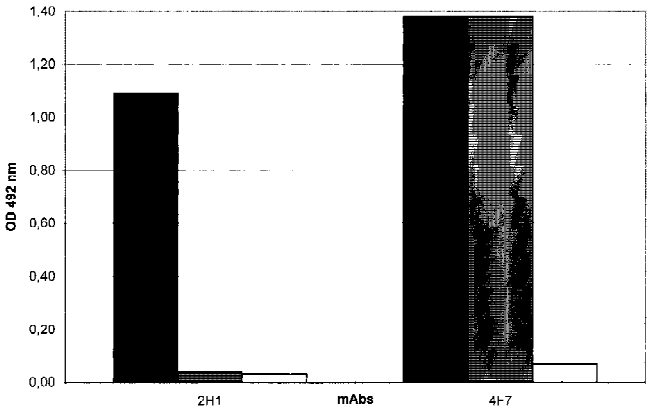

Fig. 1. Denaturation profile. Vaccinia recombinant extract RMPA95 was denatured either by boiling at 95°C for 5 min in 0.1% sodium dodecyl sulfate (SDS) or boiling for 5 min at 95°C in 0.1% SDS and 50-mM 1,4-dithiothreitol (DTT). The denatured extracts were used as solid-phase antigens in ELISA. Black bar indicates native extract; gray, extract denatured at 95°C in SDS; and white, extract denatured at 95°C in SDS and DTT.

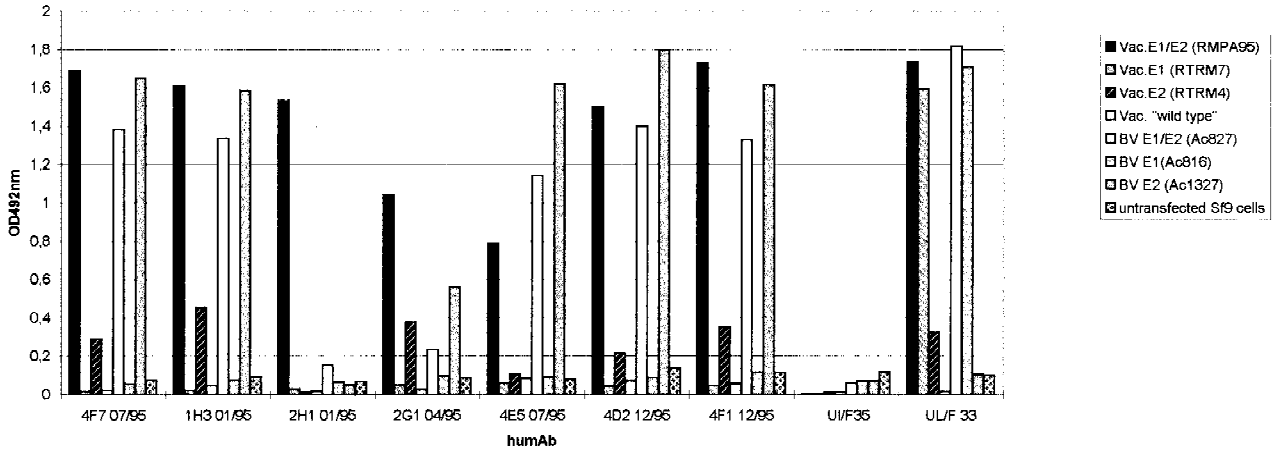

Fig. 2. ELISA with different extracts. Purified monoclonal antibodies were diluted 1:500 and tested in ELISA as described in Materials and Methods section.

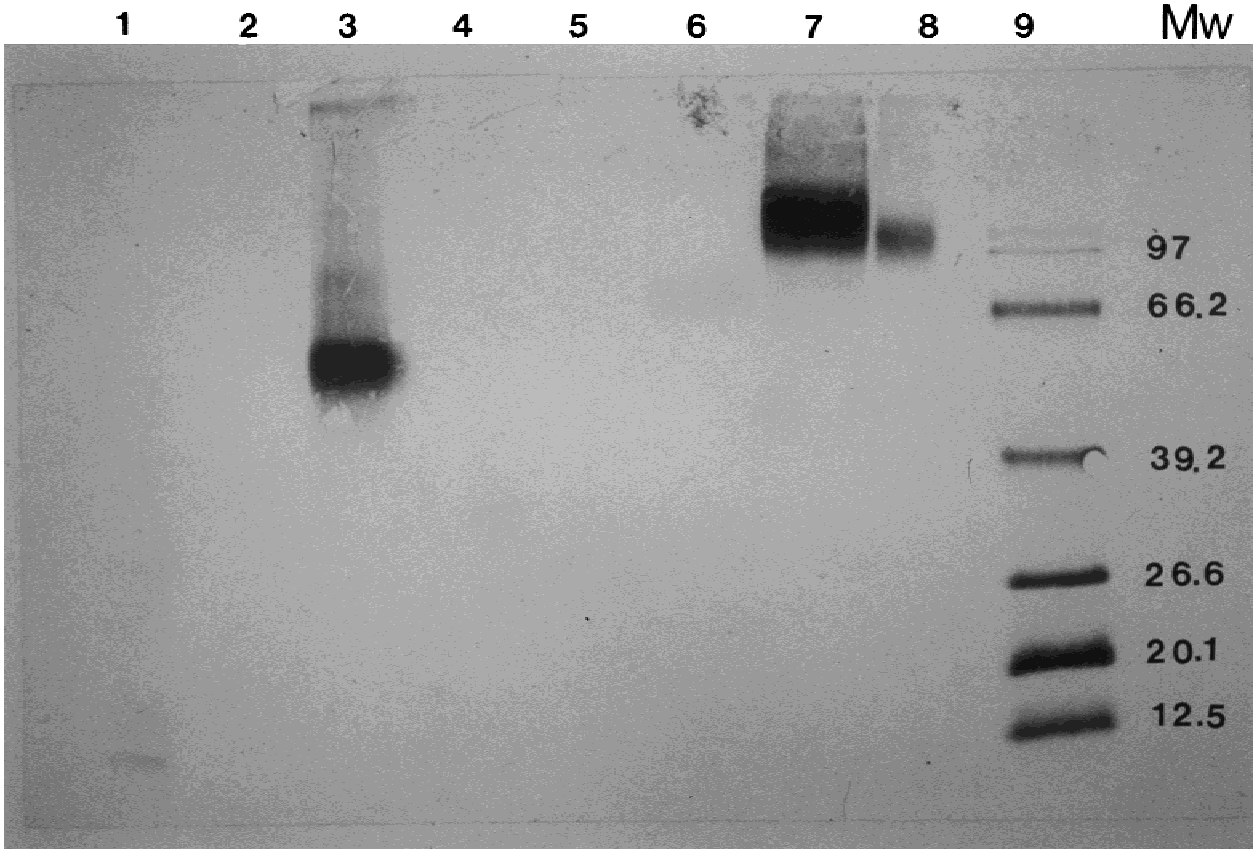

Fig. 3. Immunoblot investigation of recombinant extracts using the humAb 4F7. Detection was achieved using a monoclonal antihuman IgG biotin conjugate (Fc specific) and streptavidin/biotinylated alkaline phosphatase. Lane 1 represents prestained molecular weight marker (wide range); Lane 2, vaccinia wild-type extract; Lane 3, vaccinia RMPA95 (E1/E2) extract; Lane 4, vaccinia RTRM7 (E1) extract; Lane 5, vaccinia RTRM4 (E2) extract; Lane 6, supernatant of gD-CHO cell culture; Lane 7, supernatant of gD/E2-CHO cell culture; Lane 8, supernatant of gD/E2t-CHO cell culture; Lane 9, biotin-labeled molecular weight marker with corresponding molecular weights of the bands. Twenty micrograms of protein extract were loaded per slot.

TABLE I. Reactivity of Some humAbs to the Different Recombinant Extracts in Western Blots[a]

Cell Line ↓ Genotype →	vac E1/E2 1a	vac E2 1b	bac E1/E2 1b	bac E2 1b	CHO gD/E2 1b	CHO gDE2t 1b
1A7	+/–	negative	negative	negative	negative	negative
4D2	+/–	negative	negative	negative	positive	negative
4F1	positive	negative	negative	negative	positive	+/–
2H1	+/–	negative	negative	negative	negative	negative
1H3	positive	negative	negative	negative	positive	positive
3E2*	+/–	negative	negative	negative	negative	negative
4F7	positive	negative	negative	negative	positive	positive
4E5	+/–	negative	negative	negative	negative	negative
2G1	positive	negative	negative	negative	n.d.	n.d.
2H8[b]	positive	negative	negative	negative	positive	positive
3F12[b]	positive	negative	negative	negative	positive	positive
2F7[b]	positive	negative	negative	negative	positive	positive
2H9[b]	+/–	negative	negative	negative	n.d.	n.d.
2C4[b]	positive	negative	negative	negative	negative	negative
1B12[b]	+/–	negative	negative	negative	negative	negative
7B7[b]	positive	negative	negative	negative	positive	negative

[a]+/– denotes a very weak band in Western blot; n.d., not done.
[b]Supernatant of LCLs not yet heterohybridoma cells.

blots under nondenaturating conditions. Reactivity could only be observed with the recombinant vaccinia E1/E2 extract RMPA95, but not with the recombinant E1 or E2 extracts (RTRM7 and RTRM4, respectively). No antibody binding was obtained with the recombinant baculovirus extracts, contrasting with the results of the ELISA assay. Yet a positive signal was observed with both supernatants of recombinant CHO cells, gD/E2 and gD/E2t, but not with gD (Fig. 3). Following these results, a large number of humAbs (purified humAbs and some supernatants of LCL cultures) were further tested in Western blots. The result of this screening is listed in Table I. To assess the prevalence of antibodies to the 4F7 epitope among anti-HCV positive individuals, 38 anti-HCV/RIBA positive sera were tested in a 4F7-competitive ELISA (Fig. 4). A significant inhibition could be observed in 35 out of 38 sera tested (92%). In order to evaluate whether the isolated antibodies interfere with each other for the binding to their corresponding epitope on the E1/E2 antigen, a competitive ELISA was carried out as described above, using either monoclonal antibodies or LCL-supernatants as the competitor agent. Preincubation with humAb 1H3 inhibited the binding of 4F7 to the epitope on the E1/E2 antigen. Some inhibition to 4F7 could also be seen by humAb 4F1. The inhibition effect between 4F7 and 1H3 was confirmed in a second competitive ELISA, this time using labeled 1H3 as secondary antibody. The reciprocal inhibition with 4F7 and 4F1 could also be confirmed in this way. Using this method, we were able to identify other humAbs with reciprocal competing capacity (e.g. 2H1 and 1A7). Immunofluorescence experiments were undertaken on cell lines inducibly expressing HCV structural proteins demonstrated that the humAbs 2H1 and 4D2 may be used in immunofluorescence studies (Fig. 5). A predominantly perinuclear cytoplasmic staining pattern was found in these cells. No immunoreactivity was observed in control cells. However, further studies with

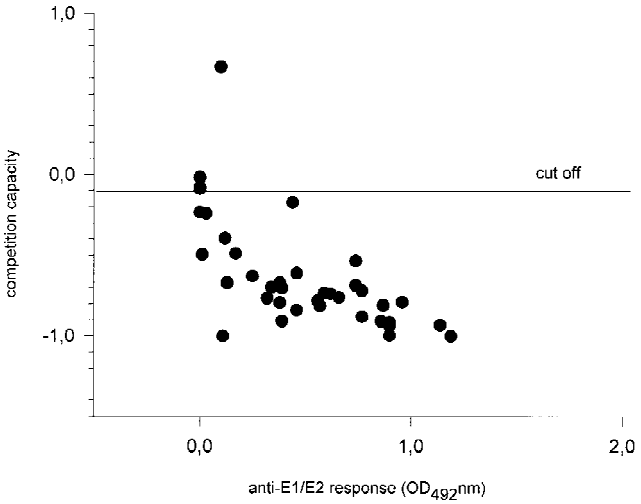

Fig. 4. Competition capacity of RIBA positive sera (n = 38) to the humAb 4F7. Test sera were added to the wells at a 1:10 dilution, followed by incubation with digoxigenin-labeled 4F7 at a 1:300 dilution. See Materials and Methods section.

4F7 using liver cryostat biopsies from patients with acute hepatitis (n = 1), chronic active hepatitis with (n = 2) and without cirrhosis (n = 2), chronic persistent hepatitis (n = 3), or hepatocellular carcinoma (n = 5) proved unsuccessful (data not shown). Data regarding the virus titer of these patients were not available.

DISCUSSION

We report the isolation of cell lines producing humAbs that reacted with recombinant HCV E1/E2 envelope protein. These cell lines were established from EBV-transformed peripheral B-cells obtained from anti-HCV positive blood donors. For the antibody screening, an antigen that was expressed in mammalian cells infected with the recombinant vaccinia virus was used. This strategy permitted polyprotein glycosylation and other posttranslational modifications of

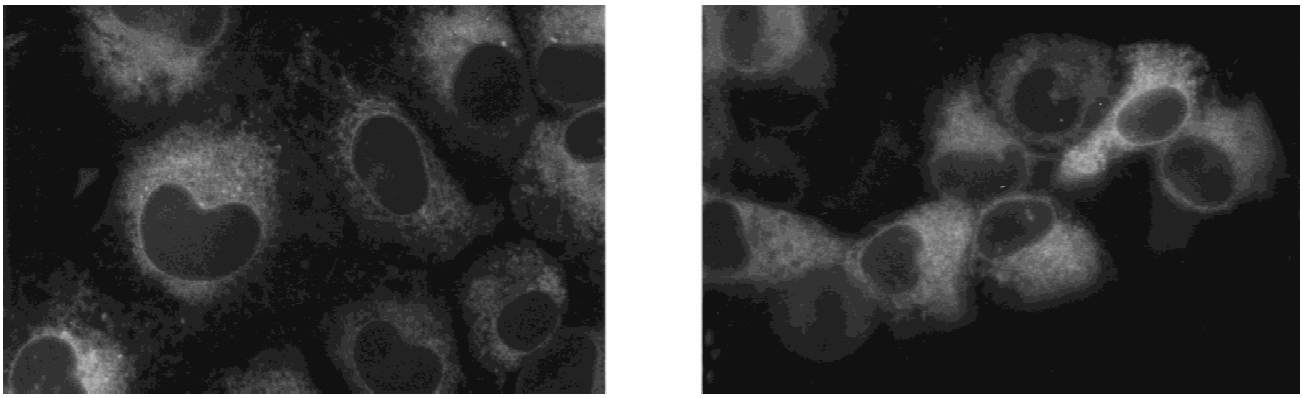

Fig. 5. Immunofluorescence investigation of cell lines inducibly expressing the HCV structural proteins (D.M., manuscript in preparation). Left: A-mAb 2H1. Right: B-mAb 4D2. Bound humAbs were detected after incubation with fluorescein isothiocyanate-conjugated rabbit F(ab′)2 fragment to human immunoglobulins °Cappel, Durham, NC).

recombinant proteins. The cloned HCV DNA sequence used to express antigens for the primary screening originated from a genotype 1a isolate.

Denaturation experiments, employing both SDS and the reducing agent DTT, demonstrated that all isolated humAbs reacted to conformation-dependent epitopes, suggesting that the used recombinant extract RMPA95 displayed a rather structural conformation. These epitopes could be further separated into two groups depending on their sensitivity to denaturation treatments. One group of antibodies reacted exclusively with the "native" protein present in the extract RMPA95 (e.g., 2H1). The second group, which includes 4F7, reacted with epitopes whose structure within the protein is maintained by disulfide bonds. The biological significance of such difference remains unknown, but this observation suggests that the isolated humAbs are not identical. This was also confirmed by competitive ELISA, where an inhibition effect was observed only between certain isolated humAbs, e.g., 4F7, 1H3, and 4F1. The use of the baculovirus extract Ac827 (E1/E2 antigen) should have permitted us to identify antibodies whose epitopes are common in both genotypes 1a and 1b. Using ELISA assays, some humAbs were identified, which reacted with the baculovirus extracts E1/E2 (Ac827) and E2 (Ac1327). However, we were not able to confirm these results by Western blots. Positive signals were obtained in Western blots using vaccinia virus extract RMPA95, CHO gD/E2, and in some cases CHO gD/E2t culture supernatants (see Table I). These results suggest that the humAbs 4D2 and 7B7, which did not react with gD/E2t antigen, may bind to an epitope at the carboxy-terminus of the HCV glycoprotein 2.

Previous work carried out by Choo et al. [1994] suggests that the use of recombinant HCV envelope antigens expressed either in yeast or insect cells failed to induce neutralizing antibodies in chimpanzees. Interestingly, the same antigens expressed in mammalian (HeLa) cells infected with a recombinant vaccinia virus induced protection in chimpanzees against HCV infection. Rosa et al. [1996] have also demonstrated that recombinant HCV glycoproteins expressed in HeLa cells were capable of binding to human cells. Since the major difference between these recombinant antigens lies in posttranslation glycosylation steps, one could speculate that glycosylation may play a critical role in the immunogenicity of the HCV envelope proteins. In insect cells, glycosylation of proteins results in less complex oligosaccharide structures than in mammalian cells. This difference could explain the absence of binding of the isolated humAbs to recombinant baculovirus extracts in Western blots. Preliminary experiments by our group have shown that deglycosylation of the vaccinia extract RMPA95 by peptide-N-glycosidase F (PNGase) abrogates 4F7 binding to antigen in Western blots (data not shown). Binding of the isolated humAbs to recombinant extracts from mammalian cells was also demonstrated by immunoblotting. These experiments suggest that humAbs 4D2, 1H3, 4F7, 2H8, 3F12, 2F7, and 7B7 are anti-E2 antibodies whose antigenic determinants are common to genotypes 1a and 1b (see Table I). The fact that none of the isolated humAbs reacted strongly with recombinant vaccinia extract RTRM4 (E2), even in ELISA assays, could be due to the low expression level of this antigen. Alternatively, there may be an absence of glycosylation of this antigen due to the lack of the signal sequence at the C-terminus of E1 [Saito et al., 1997]. This glycosylation may be required for humAbs binding.

The humAb 4F7 enabled the identification of a conserved epitope on the E2 protein. Ninety-two percent of the investigated RIBA positive blood donors had antibodies that recognized this epitope. This finding indicates a highly conserved structure, which may be valuable not only for diagnostic purposes but also as a tool in the purification and characterization of other recombinant envelope proteins. Concerning the latter, preliminary results suggest that the humAbs 4D2 and 2H1 can be successfully used in immunofluorescence microscopy of transfected cells (see Fig. 5). To date, immunohistochemical investigation of HCV-infected liver tissue has only been performed with the humAb 4F7. No positive result has been obtained. Other hum-

Abs will have to be tested before further conclusions concerning this application can be reached. However, the low concentration of HCV antigens in infected liver cells, compared with the high-level expression in transfected cells, might explain the difficulties encountered [Lau et al., 1996].

Further characterization of the monoclonal antibodies described in this work is being carried out, e.g., in vitro neutralization studies and a more detailed epitope mapping. Results will be published elsewhere.

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